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Group B *Streptococcus* Capsular Polysaccharide-Cholera Toxin B Subunit Conjugate Vaccines Prepared by Different Methods for Intranasal Immunization

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Group B *Streptococcus* (GBS) type III capsular polysaccharide (CPS III) was conjugated to recombinant cholera toxin B subunit (rCTB) using three different methods which employed (i) cystamine and *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), (ii) carbodiimide with adipic acid dihydrazide (ADH) as a spacer, or (iii) reductive amination (RA). The CPS III-rCTB conjugates were divided into large- and small-molecular-weight (M_r) fractions, and the immunogenicities of the different preparations after intranasal (i.n.) immunization were studied in mice. Both large- and small-M_r conjugates of CPS III-rCTB$_{RA}$ or CPS III-rCTB$_{ADH}$ induced high, almost comparable levels of CPS-specific immunoglobulin G (IgG) in serum, lungs, and vagina that were generally superior to those obtained with CPS III-rCTB$_{SPDP}$ conjugates or a CPS III and rCTB mixture. However, the smaller-M_r conjugates of CPS III-rCTB$_{RA}$ or CPS III-rCTB$_{ADH}$ in most cases elicited a lower anti-CPS IgA immune response than the large-M_r conjugates, and the highest anti-CPS IgA titers in both tissues and serum were obtained with the large-M_r CPS III-rCTB$_{RA}$ conjugate. Serum IgG anti-CPS titers induced by the CPS III-rCTB$_{RA}$ conjugate had high levels of specific IgG1, IgG2a, IgG2b, and IgG3 antibodies. Based on the effectiveness of RA for coupling CPS III to rCTB, RA was also tested for conjugating GBS CPS Ia with rCTB. As for the CPS III-rCTB conjugates, the immunogenicity of CPS Ia was greatly increased by conjugation to rCTB. Intranasal immunization with a combination of CPS Ia-rCTB and CPS III-rCTB conjugates was shown to induce anti-CPS Ia and III immune responses in serum and lungs that were fully comparable with the responses to immunization with the monovalent CPS Ia-rCTB or CPS III-rCTB conjugates. These results suggest that the GBS CPS III-rCTB and CPS Ia-rCTB conjugates prepared by the RA method may be used in bivalent and possibly also in multivalent mucosal GBS conjugate vaccines.

Group B *Streptococcus* (GBS) is one of the major pathogens that can be transferred to neonates from the mother through the vaginal tract and causes neonatal bacteremia, sepsis, and meningitis (13, 36, 37). Protective immunity to this organism in neonates can be achieved by maternal antibodies to the capsular polysaccharide (CPS) of GBS, which is transferred through the placenta (31). Like other bacterial CPSs, the conjugation of GBS CPS to an appropriate carrier protein, such as tetanus toxoid, may result in an increase of the immune response to CPS, probably due to both the recruitment of carrier-specific T helper cells and other as-yet-undefined mechanisms (1, 24, 30, 31, 44). The most common GBS serotypes associated with invasive disease are types III, Ia, and V. In several animal studies, these GBS serotype CPS-tetanus toxoid conjugates have been shown to be effectively protective for the offspring after systemic immunization of the mother (1, 24, 30, 44). Since colonization of the genital and lower intestinal tracts is important in transmission of GBS, effective immunity at genital and rectal mucosal sites may be necessary to diminish or eliminate the colonization of this organism, thus preventing it from spreading. In recent years, studies by several groups have shown that intranasal (i.n.) vaccination with *Streptococcus pneumoniae* CPS conjugate vaccine can not only protect mice against invasive *S. pneumoniae* infection but also effectively reduce colonization in the lungs (21, 22, 38). In previous studies, we showed that GBS CPS type III (CPS III) conjugated with the effectively mucosa-binding nontoxic B subunit of cholera toxin (CTB) using the reductive amination (RA) method could induce both strong systemic and local mucosal immune responses and also that the levels of serum antibodies correlated with the opsonizing activity (40, 41).

The efficacy of CPS-carrier protein conjugates may be influenced by several factors, such as (i) the conjugation methods used, (ii) the extent of cross-linking between the CPS and the carrier protein, (iii) the molecular weight of the conjugate, and (iv) the content of free polysaccharide in the conjugate, which has been shown to inhibit the immune responses elicited by the conjugated CPS (33, 34). For conjugation to CTB, an especially sensitive and important aspect is the preservation of the binding activity of the coupled CTB to its mucosal receptor, the GM1 ganglioside (17). However, the influence of these characteristics of CPS conjugates on their immunogenicities has not been adequately examined after mucosal vaccination.

For practical reasons, the ability to combine different conjugate vaccines in formulations that can be administered simultaneously is important to permit stimulation of protection against multiple serotypes of GBS infection within a simple

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immunization schedule. Thus, possible interactions between conjugates must be considered. It has been reported that mono- and multivalent GBS CPS conjugate vaccines can be formulated which are efficacious in inducing protective immunity in animal models by systemic immunization (32). The possibility of negative interactions in mucosal immunization with GBS CPS conjugates needs to be addressed.

In this study, we synthesized GBS CPS III-CTB conjugates with different linkage types with or without a spacer. The CPS III-CTB conjugates were fractionated into large- and small-molecular-weight batches. In addition, based on the results with the CPS III conjugates, GBS CPS Ia was also conjugated with CTB by the RA method. The anti-CPS responses were investigated after i.n. immunization with those conjugates in a mouse model to address (i) the effects of different conjugation methods of GBS CPS III with CTB, the molecule size of the conjugate, and the amount of free polysaccharide in conjugates on the anti-CPS specific immune responses; and (ii) the immunogenicity of the CPS Ia-CTB conjugate and the effect of combined immunization with CPS Ia-CTB and CPS III-CTB conjugates on the different types of anti-CPS specific immune responses.

MATERIALS AND METHODS

Chemicals. The following reagents were used: adipic acid dihydrazide (ADH) (Fluka Chimie AG, Buchs, Switzerland); avidin, cyanobromide (CNBr), 2(*N*-morpholino)ethanesulfonic acid (MES), and cystamine (all from Sigma Chemical Co., St. Louis, Mo.); dithiothreitol (DTT) (Calbiochem, La Jolla, Calif.); 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl (EDAC), *N*-hydroxysuccinimidobiotin, *o*-phenylenediamine, and sodium *m*-periodate (Sigma Chemical Co.); sodium cyanoborohydride (Aldrich Chemie, Steinheim, Germany); and *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) (Pharmacia Fine Chemicals, Uppsala, Sweden).

CPS antigens and cholera proteins. GBS CPS III was purified from the culture medium of *Streptococcus agalactiae* strain M732 as described previously (40). GBS CPS Ia was purified from *S. agalactiae* strain SS615 by the same methods used for the purification of CPS III. The purified CPS III was composed of 18 to 20% (wt/wt) sialic acid and contained <1% protein. Purified CPS Ia had a larger molecular weight than purified CPS III. It contained 13% (wt/wt) sialic acid and <0.5% (wt/wt) protein. Recombinant CTB (rCTB) was purified from culture medium of *Vibrio cholerae* strain 358 as described previously (29). Purified CT was obtained from List Biological Laboratories Inc. (Campbell, Calif.).

Preparation of CPS III-rCTB conjugate with cystamine and SPDP. To perform thiolation of CPS III using cystamine, a solution of CPS III (5 mg/ml) was activated by CNBr at pH 10.5 for 6 min at 4°C. The weight ratio of CNBr to CPS was 1.5:1. The reaction mixture was brought to pH 8.5 by 0.5 M $NaHCO_3$, and cystamine was added to a final concentration of 0.5 M. After being tumbled for 18 h at 4°C, the mixture was dialyzed against distilled water and lyophilized. The presence of NH_2 on the cystamine-derivatized CPS was verified by a 2,3,6-trinitrobenzenesulfonate (TNBS) assay (15). Cystamine-modified GBS CPS III (15 mg in 1.5 ml of 0.1 M sodium phosphate buffer containing 10 mM EDTA, pH 7.5) was reduced by 50 mM DTT. After incubation at room temperature for 4 h, the thiolated CPS III was separated from low-molecular-weight reagents by passage through a Sephadex G-25 column (Pharmacia). The SH content of the CPS III was measured by means of the Ellman test (16).

To obtain derivatization of rCTB with 2-pyridyl disulfide, SPDP was brought to a concentration of 0.4 M in absolute ethanol and then added at sevenfold molar excess to CTB in 0.1 M sodium phosphate buffer, pH 8.5. After incubation at room temperature for 1 h, the reaction mixture was passed through a Sephadex G-25 column. The fractions containing protein were stored at 4°C. The number of bound 2-pyridyl disulfide groups was estimated after reduction of an aliquot with 40 mM DTT for 1 h at room temperature and measurement of the A_{343} with an E of 8.08×10^3 M^{-1} cm^{-1} for pyridine-2-thione. The derivatized CPS III (10 mg) and rCTB (9 mg) preparations were then mixed at an equal ratio of SH groups on CPS III and 2-pyridyl disulfide groups on rCTB and left to react overnight at room temperature. The conjugate was finally purified and divided in fractions by gel filtration as described below.

Preparation of the CPS III-rCTB conjugate by EDAC with ADH as a spacer. To modify GBS CPS III with ADH as described previously (40), briefly, a solution of CPS III (5 mg/ml) was activated by CNBr at pH 10.5 for 6 min at 4°C. The weight ratio of CNBr to CPS was 1.5:1. The reaction mixture was brought to pH 8.5 by addition of 0.5 M $NaHCO_3$, and ADH was added to a final concentration of 0.25 M. After being tumbled for 18 h at 4°C, the mixture was passed through a Sephadex G-25 column with distilled water as the eluant. The fractions containing CPS were pooled, dialyzed against distilled water, and lyophilized. The presence of NH_2 on the ADH-derivatized CPS was verified by the TNBS assay.

The CPS-ADH (20 mg) and rCTB (10 mg) were mixed at a 2:1 (wt/wt) ratio in 1 ml 0.1 M MES buffer, pH 5.8, and EDAC was added to a final concentration of 0.05 M. The reaction mixture was incubated at room temperature for 4 h with tumbling, and then the conjugate was purified and divided in fractions by gel filtration as described below.

Preparation of CPS Ia- and III-rCTB conjugates by RA. Conjugation was performed as described previously (41). Briefly, GBS CPS Ia or III (20 mg) in 2 ml of phosphate-buffered saline (PBS), pH 7.0, was incubated in darkness at room temperature for 2 h with 2.75 mM sodium *m*-periodate for CPS Ia and separately for 1.5 h with 4 mM sodium *m*-periodate for CPS III. Glycerol was then added to consume any residual periodates. The mixture was passed through a Sephadex G-25 column with distilled water as the eluant and lyophilized. The oxidized CPS Ia or III (15 mg) was dissolved in 2 ml of 0.1 M sodium bicarbonate, pH 9.0, and mixed with rCTB (15 mg). Sodium cyanborohydride was added to a final concentration of 20 mg/ml, and the mixture was incubated at 37°C for 5 to 6 days. The progress of conjugation was monitored by analyzing aliquots of the mixture at various times with fast-pressure liquid chromatography (FPLC) on a Superose 6 HR 10/30 column (Pharmacia Fine Chemicals) with PBS as the eluant at a flow rate of 0.5 ml/min. Conjugation was indicated by a progressive increase in a broad high-molecular-weight protein peak monitored by measurement of UV absorbance at 280 nm. After the conjugation was completed, sodium borohydride (10 mg/ml) was added to the reaction mixture to reduce the remaining free aldehyde groups, and the conjugate was purified by gel filtration.

Purification and fractionation of conjugates. All of the conjugates were purified by gel filtration on a Sephacryl S-300 HR 16/60 column (Pharmacia Fine Chemicals) eluted with PBS to separate the conjugate from unbound rCTB. For the different CPS III-rCTB conjugates, fractions of the conjugate peak were divided into two pools according to elution volumes. The large-molecular-weight fraction pool (L) contained the elution volumes from 40 to 52 ml, and the small molecular-weight fraction pool (S) contained those from 53 to 62 ml. For the CPS Ia-rCTB conjugate, fractions corresponding to the high-molecular-weight material were pooled (elution volume, from 38 to 49 ml). The conjugates or pooled fractions were then concentrated by ultrafiltration on a Millipore membrane with a 10-kDa molecular mass cutoff.

Analyses of conjugates. To determine the relative distribution coefficient (K_{av}) of the two sizes of CPS III-rCTB conjugates, these conjugate preparations were tested by FPLC on a Superose 6HR 10/30 column equilibrated with PBS buffer.

The content of CPS was measured by means of a phenol sulfuric assay with purified CPS Ia and III as a standard (9). The content of protein was estimated by a Bio-Rad protein assay in which purified rCTB was used as the standard. The specificities of purified, modified, and conjugated CPS III were tested by immune double diffusion using rabbit immune serum to a GBS type III strain.

The immunological reactivities of CPS Ia and III and rCTB in the conjugate were determined by means of a GM1 ganglioside receptor-binding variant of an enzyme-linked immunosorbent assay (GM1 ELISA) as described previously (40). Polystyrene microwell plates (Nunc, Roskilde, Denmark) were coated overnight with monosialoganglioside (GM1) (0.3 nmol/ml). The conjugates were then added in threefold serial dilutions starting at a concentration of 2.5 μg of CPS/ml, and after incubation, a hyperimmune rabbit serum against a GBS type Ia or type III strain or a mouse monoclonal antibody to rCTB (LT 39) was added. The antibodies bound to the CPS or rCTB antigen were detected by means of alkaline phosphatase goat anti-rabbit immunoglobulin G (IgG) or horseradish peroxidase goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) conjugates and corresponding enzyme substrates, respectively. The reactivities of CPS and CTB were expressed as the lowest concentrations of the conjugates giving an absorbance of 0.4 above the background.

To estimate the amounts of unbound CPS in the CPS III-rCTB conjugates, the conjugates were adsorbed onto GM1-coated polyethylene glycol-based beads in test tubes, incubated, and shaken for 3 h at room temperature. The beads were centrifuged at 15,000 × *g*, and the supernatants were analyzed for CPS content by a phenol sulfuric assay and for rCTB content by GM1 ELISA. The GM1 beads were a gift from Jan-Erik Månsson, Department of Neurochemistry, Göteborg, Sweden.

TABLE 1. Synthesis and characteristics of GBS CPS III-rCTB conjugates

Conjugate	Coupling method	Molecular size (K_{av})	CPS/protein (wt/wt) ratio	Reactivity with antiserum (concn[a] [ng/ml])		% (wt/wt) free CPS
				CPS	CTB	
Large						
III-rCTB$_{SPDP}$ (L)	SPDP	0.20	0.91	2.0	3.5	7.4
III-rCTB$_{ADH}$ (L)	ADH	0.21	0.95	2.0	5.2	18.1
III-rCTB$_{RA}$ (L)	RA	0.20	0.90	3.2	2.2	16.2
Small						
III-rCTB$_{SPDP}$ (S)	SPDP	0.35	1.09	1.3	3.6	24.1
III-rCTB$_{ADH}$ (S)	ADH	0.36	1.52	1.6	5.2	66.6
III-rCTB$_{RA}$ (S)	RA	0.37	1.52	6.3	4.1	49.1

[a] Lowest concentration giving an absorbance of 0.4 above background in GM1 ELISA with antibodies to CPS and rCTB.

Immunization of mice. C57BL/6 female mice, 8 to 10 weeks old, were obtained from B&K Universal (Stockholm, Sweden, and Bomholtsgård, Denmark). For the studies of the immunogenicities of the different CPS III-rCTB conjugates, the conjugates were tested after division into L and small S molecular weight preparations. A control group was immunized with a mixture of 30 μg of free CPS III and rCTB. To evaluate the immunogenicity of CPS Ia conjugate and the effect of the combination with CPS III-rCTB, four groups of mice were immunized with (i) purified CPS Ia alone, (ii) CPS Ia-rCTB conjugate, (iii) CPS III-rCTB conjugate, or (iv) CPS Ia-rCTB with CPS III-rCTB conjugates. Four or five mice per group were immunized i.n. with 30 μg of CPS of the conjugates plus 1.5 μg of CT per dose at 0 to 2, 14 to 16, and 28 to 30 days. Each dose was divided and given on three consecutive days with a micropipettor with a volume of 20 to 30 μl, since our previous work has shown that this gives rise to an immune response stronger than that achieved with a single bolus administration (41). The animals were lightly anesthetized with methoxyflurane (Schering-Plough Animal Health Corp., Union, N.J.) for all immunizations. Blood samples were taken from the tail vein before immunization. The animals were euthanized 7 to 10 days after the last immunization, and blood was drawn from the subclavian vein. Perfusion-extraction with saponin was used to obtain lungs and vaginal specimens for antibody detection as previously described (40, 41).

Serologic studies. Antibodies to GBS CPS Ia and III were estimated by ELISA using biotinylated CPS Ia and III as antigens (42). Plates (Greiner, Frickenhausen, Germany) were coated with avidin (5 μg/ml) overnight and then incubated with 2 μg of biotinylated GBS CPS Ia (North American Biologicals Inc., Rockville, Md.) or III/ml for 4 h at room temperature. The tested samples were added in threefold serial dilutions and incubated overnight. A pool of positive serum from mice immunized with CPS Ia-rCTB or CPS III-rCTB conjugate was used as a positive control. Horseradish peroxidase-labeled goat antibodies to mouse IgG (Jackson ImmunoResearch Laboratories, Inc.), IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA (Southern Biotechnology Associates, Inc., Birmingham, Ala.) were used, and the ELISA was developed with *o*-phenylenediamine and H_2O_2. The antibody titers were expressed as the reciprocal dilutions of specimens giving an absorbance of 0.4 above background.

Statistics. The geometric mean (GM), standard deviation (SD), and standard error of the mean (SEM) were calculated. Student's *t* test with Bonferroni correction was used to compare mean values in different groups of mice. Statistical significance was defined as a P of <0.05.

RESULTS

Characterization of the CPS III-rCTB conjugates. GBS CPS III was coupled to the rCTB by three different conjugation methods. The conjugate preparations obtained with cystamine and SPDP, or ADH and EDAC, or reductive amination in the presence of sodium cyanoborohydride were designated III-rCTB$_{SPDP}$, III-rCTB$_{ADH}$, and III-rCTB$_{RA}$, respectively (Table 1). The conjugates were gel filtered on an S-300 HR Sephacryl column after synthesis, and their elution profiles were found to be similar (Fig. 1). Each conjugate was divided into L and S material. Analyses by FPLC on a Superose 6HR 10/30 column showed that the K_{av}s were 0.20 to 0.21 for the L and 0.35 to 0.37 for S preparations, and the estimated molecular masses by comparison with globulin proteins ranged between 500 and 5,000 kDa for L and 400 and 500 kDa for S (Fig. 2). The different L conjugates had similar CPS/protein ratios (0.9 to 1 to 0.95 to 1), whereas CPS III-rCTB$_{SPDP}$ (S) had a lower CPS/protein ratio (1.09 to 1). These and various other characteristics of the different conjugates are described in Table 1.

Immune double-diffusion examination of purified, modified, and conjugated CPS III gave an identity precipitation line when tested with hyperimmune rabbit serum against a GBS type III strain (not shown). The immunologic reactivity of the conjugated CPS was also analyzed by GM1-ELISA using the same immune serum. The conjugated CPS, in concentrations down to 1.3 to 6.3 ng/ml for the different conjugates, reacted with the anti-GBS type III immune serum. GM1-ELISA was also used to study the receptor-binding capacity and immunologic reactivity of the conjugated rCTB. The different conjugates, in concentrations tested down to 2.2 to 5.2 ng/ml, reacted with an anti-CTB mouse monoclonal antibody. This reactivity was similar to that of unconjugated rCTB and indicates that the conjugated rCTB had essentially the same GM1-binding capacity and immunologic reactivity as unconjugated rCTB. There were no obvious differences between L and S molecular weight conjugates with regard to immunological or GM1-binding reactivities (Table 1).

Immunogenicity of different CPS III-rCTB conjugates and L and S fractions. (i) CPS III antibodies in serum. The postimmunization sera from the group of mice immunized with a mixture of free CPS III and rCTB contained levels of anti-CPS III specific IgM, IgG, and IgA antibodies that were three- to fivefold higher than the preimmunization level (Table 2). Surprisingly, immunization with both the III-rCTB$_{SPDP}$ (L) and (S) conjugates elicited only levels of IgM-, IgG-, and IgA-specific antibodies similar to those obtained with the CPS III and rCTB mixture (Table 2). In contrast, both the III-rCTB$_{AHD}$ and III-rCTB$_{RA}$ conjugates induced significantly higher levels of serum IgG and IgA antibodies than the III-rCTB$_{SPDP}$ conjugate [III-rCTB$_{RA}$ (L) versus III-rCTB$_{SPDP}$ (L); $P < 0.001$; III-rCTB$_{ADH}$ (L) versus III-rCTB$_{SPDP}$ (L), $P < 0.01$] (Table 2). Immunization with the smaller-M_r III-rCTB$_{ADH}$ (S) and III-rCTB$_{RA}$ (S) conjugates induced levels of serum IgG antibodies similar to those of the large-M_r conjugates prepared by the same methods, but the levels of serum IgA antibodies were significantly lower than those obtained with the L conjugates ($P < 0.05$ and $P < 0.01$, respectively).

(ii) IgG subclasses. The levels of serum anti-CPS III IgG subclasses induced by the III-rCTB$_{RA}$ (L) and (S) conjugates were tested and compared with those induced by CPS mixed with rCTB. The III-rCTB$_{RA}$ (L) conjugate induced significantly higher levels of anti-CPS serum IgG1, IgG2a, IgG2b, and IgG3 antibodies than the CPS III and rCTB mixture (Fig. 3). There were no significant differences in the levels of IgG1, IgG2b, and IgG3 antibodies induced by the S conjugate and the L conjugate, whereas the level of IgG2a after immunization with III-rCTB$_{RA}$ (S) was much lower than that obtained with the L conjugate ($P = 0.004$).

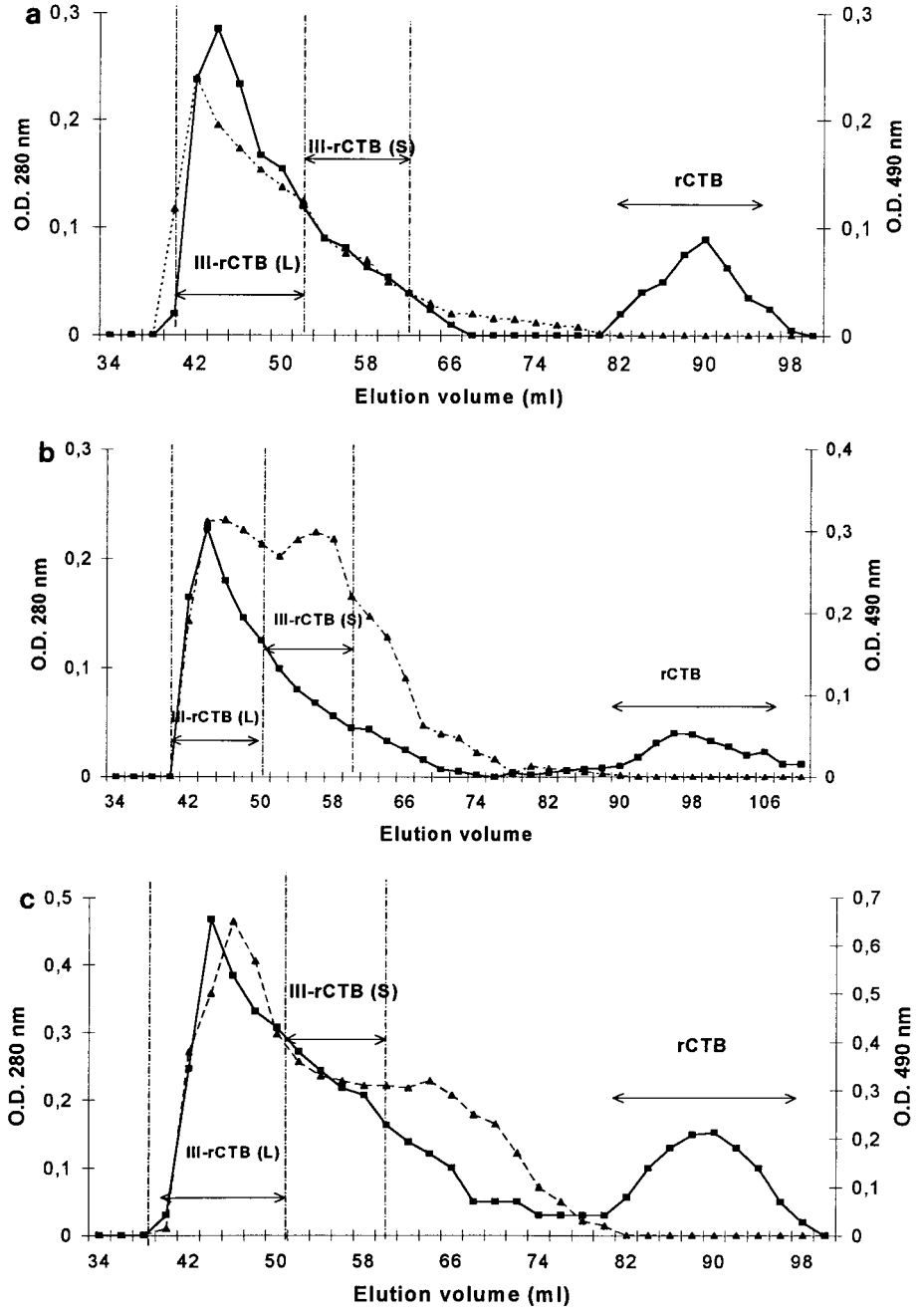

FIG. 1. Gel filtration of GBS type III CPS-rCTB reaction mixture on the Sephacryl S-300 HR 16/60 column after conjugation. (a) III-rCTB$_{SPDP}$; (b) III-rCTB$_{ADH}$; (c) III-rCTB$_{RA}$ conjugates. The flow rate was 19.8 ml/h, and the fraction size was 1 ml. The CPS was monitored by the phenol sulfuric assay (optical density [O.D.] at 490 nm (▲), and the protein was monitored by the optical density at 280 nm (■). The peaks containing the III-rCTB conjugate were divided into L (elution volume, 40 to 52 ml) and S (elution volume, 53 to 62 ml) M_r pools.

CPS III antibodies in lungs and vagina. The anti-CPS III IgG and IgA titers in lungs and vaginal tissues elicited by the L conjugates prepared with different coupling reagents were evaluated. The perfusion-extraction technique used for preparing the tissue extract specimens analyzed has been shown to remove >97% of serum antibodies from the lungs and >95% from the vaginal tissues (23). In the lungs, the conjugate III-rCTB$_{SPDP}$ (L) induced only low levels of IgG and IgA anti-CPS antibodies, similar to the levels obtained with the CPS III and rCTB mixture. In contrast, the III-rCTB$_{AHD}$ (L) conjugate and, even more pronounced, the III-rCTB$_{RA}$ (L) conjugate induced a significantly higher anti-CPS III IgG and IgA response than the CPS III and rCTB mixture did (Fig. 4a). Among three types of conjugate, III-rCTB$_{RA}$ (L) induced the highest levels of anti-CPS III antibodies, both in IgG (III-rCTB$_{RA}$ versus III-rCTB$_{SPDP}$ and III-rCTB$_{AHD}$; $P < 0.001$) and in IgA (III-rCTB$_{RA}$ versus III-rCTB$_{SPDP}$ and III-rCTB$_{AHD}$; $P < 0.05$).

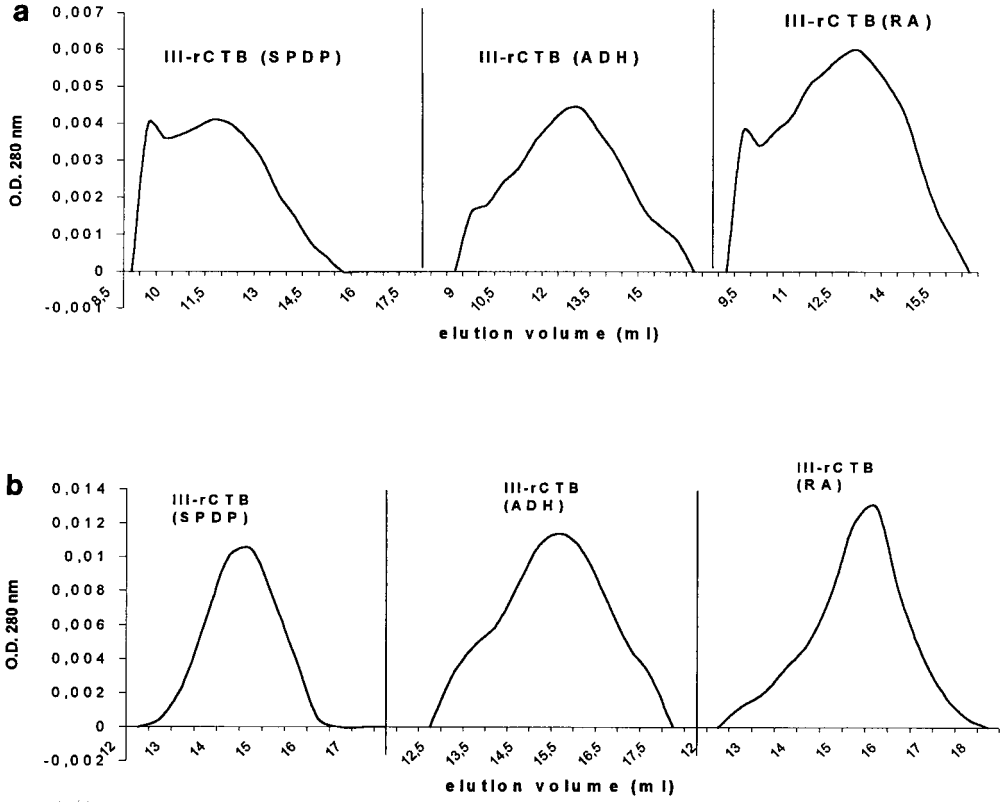

FIG. 2. Elution profile (optical density [O.D.] at 280 nm) of GBS CPS III-rCTB conjugates on Superose 6HR 10/30 gel filtration column. The larger-M_r conjugates, III-rCTB$_{SPDP}$, III-rCTB$_{ADH}$, and III-rCTB$_{RA}$ (a), had peak K_{av} values of 0.2, 0.21, and 0.2, respectively; the smaller-M_r conjugates, III-rCTB$_{SPDP}$, III-rCTB$_{ADH}$, and III-rCTB$_{RA}$ (b), had peak K_{av} values of 0.35, 0.36, and 0.37, respectively. The void and bed volumes of the column were 8.8 and 25 ml, respectively.

In the vagina, the III-rCTB$_{ADH}$ (L) and III-rCTB$_{RA}$ conjugates were also found to induce significantly higher IgG and IgA responses than the CPS III and rCTB mixture did (Fig. 4b). The highest titers of both IgG and IgA were still found following i.n. immunization with III-rCTB$_{RA}$ (L) (for IgG, III-rCTB$_{RA}$ versus III-rCTB$_{SPDP}$, $P < 0.001$, and versus III-rCTB$_{AHD}$, $P = 0.0018$; for IgA, III-rCTB$_{RA}$ versus III-rCTB$_{SPDP}$, $P = 0.0078$). These findings indicate that the coupling method of conjugates also influenced the mucosal specific immune responses.

When the anti-CPS III responses in lungs and vagina tissues elicited by small-M_r conjugates were analyzed, there was no significant difference between L and S conjugates prepared by the same method in the induction of the CPS III-specific IgG and IgA responses in the lungs. All of the S conjugates could induce levels of vaginal specific IgG similar to those induced by the corresponding L conjugates. However, the IgA titer in the vagina elicited by III-rCTB$_{RA}$ (S) was nearly five times lower than that after immunization with III-rCTB$_{RA}$ (L) ($P = 0.0078$).

Preparation and testing of GBS CPS Ia-rCTB conjugate alone and in combination with CPS III-rCTB conjugate. As described above, the results with CPS III indicated strongly that conjugation to rCTB by RA and isolating the L fraction yielded the most immunogenic vaccine for i.n. immunization.

TABLE 2. Serum anti-GBS CPS III antibody responses after i.n. immunization with CPS III-rCTB conjugates

Antigen	Serum anti-CPS III titer (GM ± SD) (reciprocal)					
	IgM	*P* value[a] (<0.05)	IgG	*P* value[a] (<0.05)	IgA	*P* value[a] (<0.05)
CPS III + rCTB	817 (579–1,154)		460 (400–530)		18 (7–48)	
III-rCTB$_{SPDP}$ (L)	714 (552–923)		696 (537–891)		17 (6–43)	
III-rCTB$_{SPDP}$ (S)	782 (451–1,355)		104 (23–461)		41 (32–51)	
III-rCTB$_{ADH}$ (L)	2,299 (1,457–3,628)*	$P = 0.007$	2,764 (1,698–4,466)‡‡	$P < 0.0001$	270 (74–977)‡	$P = 0.01$
III-rCTB$_{ADH}$ (S)	708 (495–1,012)†		1,508 (935–2,430)	$P = 0.002$	43 (24–77)§	
III-rCTB$_{RA}$ (L)	1,073 (746–1,544)		39,036 (19,498–77,624)§§	$P < 0.0001$	1,234 (1,099–1,384)**	$P = 0.0001$
III-rCTB$_{RA}$ (S)	1,332 (933–1,901)		27,195 (15,075–49,058)	$P < 0.0001$	79 (23–268)††	
Preimmunization	150 (92–205)		145 (70–201)		<10	

[a] *P* values are given for antibody titers which are significantly different from those in the group of mice immunized with CPS plus rCTB. * versus †, $P < 0.01$; ‡ versus §, $P < 0.05$; ** versus ††, $P < 0.01$; ‡‡ versus §§, $P < 0.001$; ‡ versus **, $P < 0.01$.

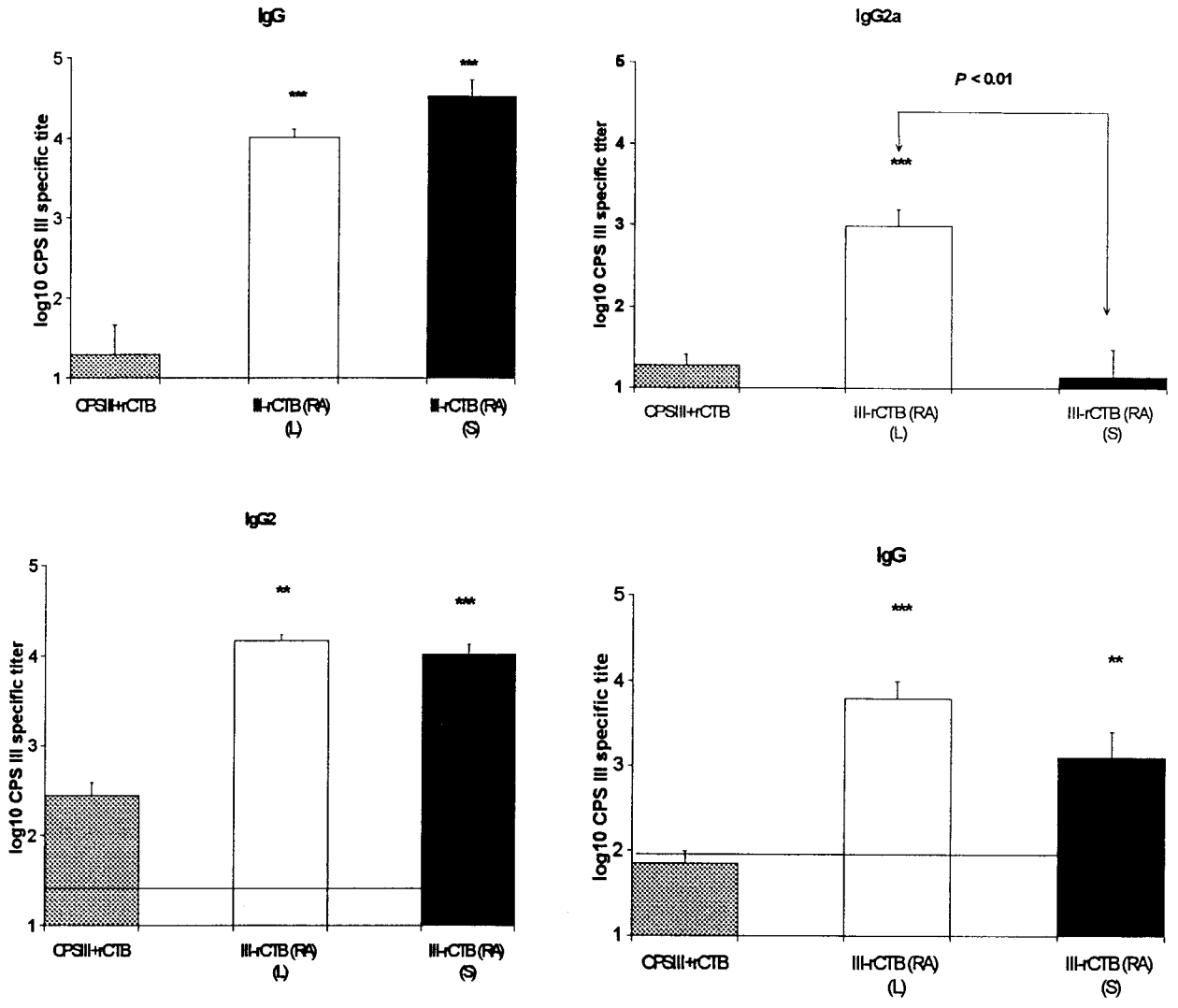

FIG. 3. Serum anti-CPS specific IgG subclass titers after i.n. immunization with large- and small-M_r III-rCTB$_{RA}$ conjugates. Antibody titers are given as $\log_{10}$ of the GM titer + SEM. The horizontal line indicates maximal titers seen in the preimmunization samples (0.7 $\log_{10}$ for IgG1 and IgG2a, 1.4 $\log_{10}$ for IgG2b, and 2.0 $\log_{10}$ for IgG3). The open bars represent the titers of large-M_r conjugates, the solid bars represent titers of small-M_r conjugates, and the shaded bars represent titers of a CPS III and rCTB mixture. Statistically significant differences are shown as follows: ***, $P < 0.001$, and **, $P < 0.01$ (compared with the CPS III and rCTB mixture group).

We wished to examine whether the RA method would also work well for conjugating other GBS CPSs to rCTB and tested this with CPS Ia.

(i) Characterization of Ia-rCTB$_{RA}$ conjugate. A small amount of the oxidized CPS Ia was found to be insoluble, and after the conjugation reaction was completed, there was also a small amount of insoluble material in the Ia-rCTB$_{RA}$ conjugate preparation. The gel filtration profile of the Ia-rCTB$_{RA}$ conjugate showed that a major portion of the rCTB was conjugated to CPS Ia (Fig. 5). The fractions containing the largest-M_r material (the void volume) were collected in order to avoid unconjugated CPS and rCTB. The ratio (wt/wt) of CPS Ia and rCTB in the resulting final conjugate was found to be 0.83 to 1, and the yield of total CPS recovered in the conjugate was 16%. The conjugated CPS Ia, in concentrations down to 10 ng/ml, reacted with anti-GBS type Ia hyperimmune rabbit serum, and the conjugated rCTB, down to a concentration of 8 ng/ml, reacted with the anti-CTB mouse monoclonal antibody (not shown). The results indicate that the conjugate had the expected in vitro immunologic and receptor-binding properties.

(ii) Immunogenicity of CPS Ia-rCTB conjugate and effect of combination immunization. Similar to the case for the CPS III-rCTB conjugate, the CPS Ia-rCTB conjugate induced higher levels of serum IgG and IgA antibodies than did unconjugated CPS Ia ($P = 0.005$ and $P < 0.05$, respectively). After i.n. immunization with the CPS Ia-rCTB conjugate, a 15-fold-higher level of specific IgG ($P < 0.01$) and a 6-fold increase in specific IgA were recorded in the lungs, compared with purified CPS Ia alone (Table 3). Mucosal immunization with a combination of CPS Ia-rCTB and CPS III-rCTB conjugates did not affect the immune responses to the CPS Ia and III antigens compared to immunization with each conjugate alone ($P > 0.10$ for all titers measured). Thus, the antibody levels

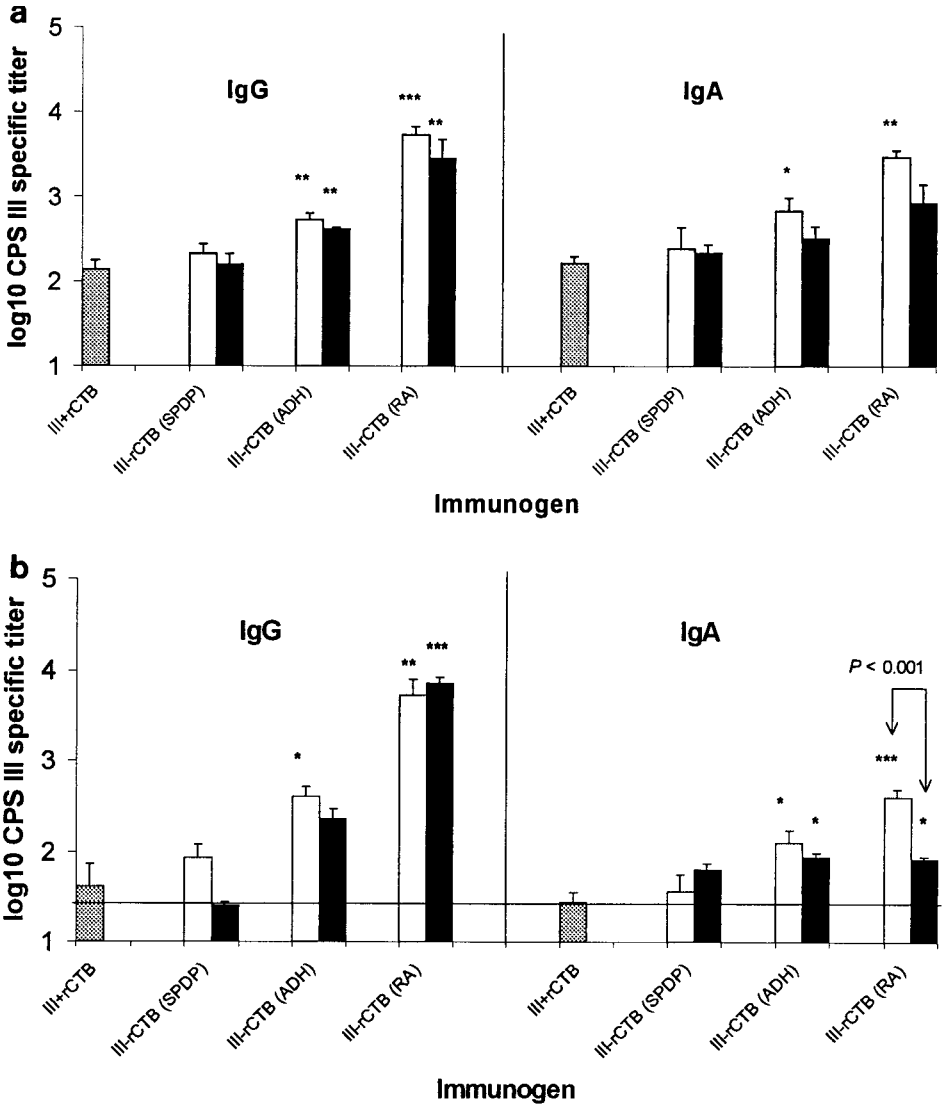

FIG. 4. Anti-CPS specific IgG and IgA in lungs (a) and vagina (b) after i.n. immunization with large- and small-M_r CPS III-rCTB conjugates. Antibody titers are given as $\log_{10}$ of the GM titer + SEM. The horizontal line indicates maximal titers seen in the PBS control group (0.95 $\log_{10}$ in lungs and 1.4 $\log_{10}$ in vagina). The open bars represent the titers of larger-M_r conjugates, the solid bars represent titers of small-M_r conjugates, and the shaded bars represent titers of a CPS III and rCTB mixture. Statistically significant differences are shown as follows: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ (compared with the CPS III and rCTB mixture group).

achieved in both serum and lungs with the combination were fully comparable to those achieved with immunization with the monovalent CPS Ia-rCTB or CPS III-rCTB conjugate separately (Table 3).

DISCUSSION

The ideal vaccine against GBS infections should stimulate both local mucosal and systemic immunities. In vaccinated women, the role of mucosal antibodies would be to prevent colonization of the female genital tract and possibly also to defend the respiratory tract of the newborn, while the systemic humoral immunity via transplacental transfer of IgG antibodies could protect the neonate when bacteria reach the bloodstream.

This study was undertaken with the long-term aim of developing a multivalent vaccine against GBS based on a cocktail of selected serotypes of CPS conjugated to rCTB and to be administered i.n. in order to stimulate both mucosal and systemic immunity. This approach has been taken based on several considerations. Secretory antibodies against CPS have been found to inhibit colonization by a capsulated pathogen, *Haemophilus influenzae* type b, in an infant rat model (25). It was also shown, both in mouse models and in humans, that immunization or colonization with GBS in the rectum or uterine cervix or even the nasal cavity could induce cervicovaginal IgA antibodies to GBS (18, 19). I.n. immunization has been found to be superior to other routes for inducing local immunity in the respiratory and genital tracts together with a strong serum antibody response (3–6, 20, 23). Finally, CTB has been proved to be an effective mucosal carrier protein for several conjugated polysaccharide antigens; including dextran (3), *H. influ-*

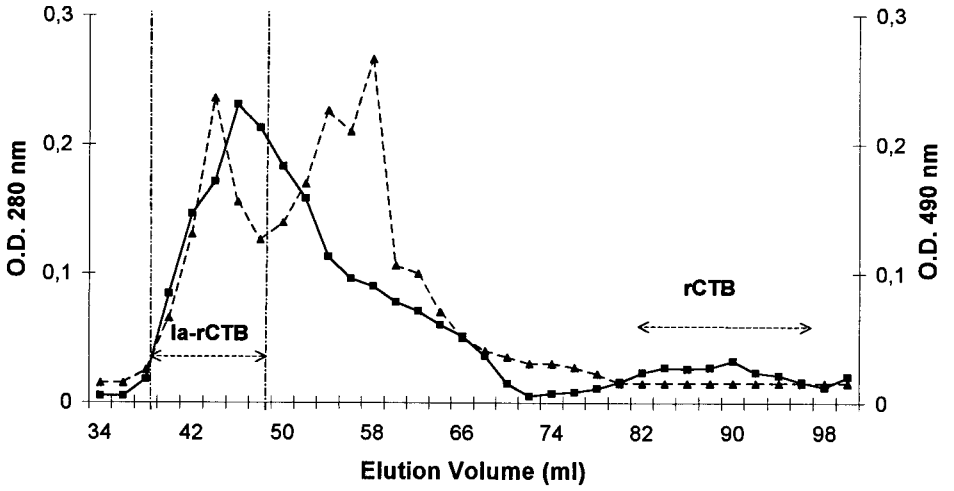

FIG. 5. Gel filtration of GBS CPS Ia-rCTB reaction mixture on the Sephacryl S-300 HR 16/60 column after conjugation. The flow rate was 19.8 ml/h, and the fraction size was 1 ml. The CPS was monitored by the phenol sulfuric assay (optical density [O.D.] at 490 nm [▲]), and the protein was monitored by the optical density at 280 nm (■). The peak containing a large-M_r CPS Ia-rCTB conjugate was pooled (elution volume, 38 to 49 ml).

enzae type b CPS (4), and recently also GBS type III CPS (40, 41).

It has been reported that GBS CPS III conjugated with tetanus toxoid by means of RA could induce high levels of anti-CPS antibody in serum after systemic immunization in both mice and human trials (24, 30). However, parenteral immunization with the GBS CPS III conjugate was unable to stimulate any significant mucosal anti-CPS response (40). In contrast, our previous studies showed that GBS CPS III coupled to rCTB by the RA method and given by i.n. immunization could effectively elicit strong T-cell-dependent immune responses both in serum and at mucosal sites (40, 41). In this study, we show by comparison with two other conjugation methods that GBS CPS-rCTB conjugate prepared by RA is the most immunogenic and is especially superior when the isolated large-molecular-weigh fraction (L) is used as a vaccine.

The three large-M_r conjugates with identical CPS and carrier protein components and similar molecule weights and ratios of CPS to protein exhibited significant disparity in immunogenicity. It has been shown that when bacterial CPS was conjugated to a carrier protein via an ADH linkage, immunogenicity could be greatly enhanced (7, 11, 12, 26–28, 40). Consistent with this, in the present study, the GBS III-rCTB$_{ADH}$ (L) conjugate could induce substantial levels of both IgG and IgA antibodies in serum, lungs, and vagina. However, the magnitude of the response induced by this conjugate was smaller than that induced by III-rCTB$_{RA}$ (L), both in serum and organs. This difference may be due to the more precise or effective coupling of CPS to rCTB by RA. Compared with the more random coupling of GBS CPS to rCTB via ADH, the conjugation using RA permits the CPS III-terminal ends of sialic acid residues to be exposed to the immune system effectively by specifically coupling the side chain terminal of CPS to rCTB. In addition, it has been reported that conjugation with ADH as a spacer was difficult to standardize, often resulting in conjugates with highly variable immunogencities (39).

In this study, even the large-M_r CPS III-rCTB conjugate prepared with SPDP failed to evoke more than marginal IgG or IgA antibody responses, even though this conjugate had a relatively small amount of free CPS and a molecular mass similar to that of the strongly immunogenic ADH (L) and RA (L) conjugates. The exact reason for this discrepancy is unclear. It has been reported that the SPDP disulfide bond of the conjugate can be unstable and have a short life in vivo (11). It is possible that degradation of the III-rCTB$_{SPDP}$ conjugate by respiratory tract enzymes might have caused the low levels of specific responses after i.n. immunization. On the other hand, SPDP-prepared conjugates between protein antigens and rCTB

TABLE 3. Effect of bivalent CPS Ia and III conjugate combinations administered i.n. on anti-CPS immune responses

Immunogen	Antibody titer (GM ± SD) (reciprocal)			
	Serum		Lungs	
	IgG	IgA	IgG	IgA
Anti-CPS Ia antibodies				
CPS Ia	536 (289–991)	32 (12–58)	25 (12–52)	13 (5–34)
Ia-rCTB	6,368 (2,110–19,217)	224 (37–1347)	382 (106–1,381)	82 (21–319)
Ia-rCTB and III-rCTB	4,225 (2,531–7,054)	186 (97–357)	223 (150–330)	81 (37–176)
Anti-CPS III antibodies				
III-rCTB	29,620 (18,669–49,997)	1,639 (519–5,171)	1,744 (708–4,295)	626 (280–1,397)
Ia-rCTB and III-rCTB	34,640 (26,001–41,209)	1,786 (1,333–2,371)	1,973 (1,238–3,019)	733 (457–1,148)

have been stably immunogenic when given i.n. (23), and also other polysaccharide-rCTB conjugates using SPDP coupling have worked well (3).

It is known that glycoconjugate vaccines generally induce stronger anti-polysaccharide antibody responses with a broader isotype range, which mainly consist of IgM and IgG1 antibodies in mice (8), than those obtained with polysaccharide alone. In this study, i.n. immunization with the III-rCTB$_{RA}$ (L) conjugate induced not only high levels of anti-CPS specific IgG1, IgG2b, and IgG3, but also a substantial level of IgG2a antibodies in serum. It has been shown that IgG2a and IgG3 are able to fix complement and promote opsonophagocytosis effectively in mice (10, 14, 35). Thus, the wider range of anti-CPS antibody isotypes after i.n. immunization with III-rCTB$_{RA}$ (L) conjugates may have a significant functional impact on the extent of protection against encapsulated GBS infection.

Generally, large-M_r conjugates are more immunogenic than smaller ones in inducing anti-CPS serum antibody responses following parenteral immunization (45). The presence of free CPS in conjugate formulations is known to suppress the T-helper cell-dependent anti-CPS response after systemic immunization (2, 43). In the present study, the small-M_r conjugates prepared with either ADH or RA contained 50 and 66% free CPS, respectively. These small-M_r conjugates could induce a level of IgG antibodies in serum, lungs, and vagina similar to that induced by the large-M_r conjugates, which contained 15 to 20% free CPS. Thus, there was no significant suppression by the larger amount of free CPS in the smaller-M_r conjugates. However, these small-M_r conjugates generally elicited a lower level of specific IgA antibodies in serum and vagina than the large-M_r conjugates. In addition, a disparate pattern of serum IgG subclasses was found after i.n. immunization with the two conjugates of different molecular sizes, with a significantly lower level of specific IgG2a being induced by III-rCTB$_{RA}$ (S) than by a large-M_r conjugate. This finding suggests that the molecular size of a conjugate and the amount of free CPS in a conjugate may influence the qualitative characteristics of the antibody response achieved.

Since the GBS CPS III-rCTB conjugate prepared with RA could most effectively improve the immunogenicity of CPS III, we coupled GBS CPS Ia to rCTB by the same method. Our result demonstrated improved immunogenicity of rCTB-conjugated CPS Ia compared with that of free CPS Ia after i.n. immunization. However, the immune responses to the conjugated CPS Ia did not achieve the same high antibody levels noted for the immune responses to the conjugated CPS III, either in serum or lungs. This might be due to the fact that CPS Ia is a larger-M_r polysaccharide than CPS III and also, or alternatively, the fact that there is a higher percentage of oxidation of CPS Ia, and therefore more extensively cross-linked conjugates (1, 45) may form insoluble material. Still, the Ia-rCTB$_{RA}$ conjugate was impressively immunogenic compared with the unconjugated CPS Ia, and most importantly, combining i.n. immunization with CPS Ia-rCTB and CPS III-rCTB had no negative effect on either the type Ia or the type III antibody responses in serum and lungs.

To summarize, GBS CPS III-rCTB conjugates obtained by different coupling methods have different effects on evoking anti-CPS specific systemic and mucosal responses after i.n. immunization. The molecular sizes of the conjugates and the presence of free CPS do not greatly affect the specific IgG isotype immune response, but these parameters may affect the serum IgG subclass distribution and have a negative effect on the mucosal IgA antibody levels achieved. The GBS CPS Ia-rCTB and CPS III-rCTB conjugates prepared by the RA method show promise as a bivalent, or component of a multivalent, mucosal conjugate vaccine against GBS infection.

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